ECOLOGICAL EFFICACY OF CHEMICALLY-MEDIATED ANTIPREDATOR DEFENSES IN THE EASTERN NEWT, *NOTOPHTHALMUS VIRIDESCENS*

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This thesis is dedicated to my parents, my friends, and Loki, the happiest field assistant on earth. Without their help, this thesis—and my scientific career—would not have happened.

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SUMMARY

Frogs, toads, and salamanders are well known for harboring an array of distasteful (and poisonous) secondary metabolites, presumably as antipredator defenses; yet few experiments have rigorously demonstrated the efficacy of amphibian chemical defenses against ecologically relevant consumers. For example, despite an absence of rigorous statistical evidence showing their distastefulness to predators, eastern newts (*Notophthalmus viridescens* (Rafinesque))—a common salamander in lentic North American habitats—are assumed to tolerate diverse predator assemblages because newts secrete tetrodotoxin (TTX), a neurotoxin. Here we combine laboratory and field-based ecology with bioassay-guided separation of chemical extracts to show that eastern newts—although chemically protected against ecologically important consumers in lentic systems—nonetheless suffer substantial predation when tethered in the field. When offered newts with alternative prey (paedomorphic *Ambystoma talpoideum*), red swamp crayfish (*Procambarus clarkii*) and largemouth bass (*Micropterus salmoides*) were 9–10$\times$ as likely to feed on *A. talpoideum* as newts. Additionally, juvenile bluegill (*Lepomis machrochirus*) were 70% less likely to consume newt eggs compared to control food pellets. We also show that different newt tissues were differentially palatable to predatory fish. All bluegill tested consumed a palatable control food, but only 20% consumed dorsal skin, only 35% ate ventral skin, but 75% fed on newt viscera, suggesting that deterrent metabolites are concentrated in the skin. Bioassay-guided fractionation revealed that crude and water-soluble newt chemical extracts inhibited bluegill feeding, definitively establishing the chemical nature of newt antipredator defenses, although we were unsuccessful at isolating the chemical compounds

responsible for unpalatability. Yet, deterrent activity in the polar but not the lipophilic chemical fraction and bioassay results demonstrating that naïve predators rapidly learn to avoid natural concentrations of TTX support the possible role of TTX in suppressing predation on newts. However, when tethered in the field, newt mortality was 55% higher in ponds with predatory fishes than in ponds lacking fishes (62% vs. 40% respectively), indicating the possible existence of other predators that are resistant to (or tolerant of) newt chemical defenses. Together, these results stress the importance of rigorous, ecologically relevant, and hypothesis-driven experimentation to better understand the complexity of chemically-mediated predator-prey interactions, even for well-studied species like *N. viridescens*.

CHAPTER I

INTRODUCTION

Predation plays a key role determining the composition and structure of communities (Paine, 1966; Morin *et al.*, 1983; Post *et al.*, 2008) and in shaping the evolutionary trajectories of prey (Vermeij, 1994; Hanifin *et al.*, 1999). Predators can reduce prey abundances directly through consumption (Murdoch *et al.*, 2003) and reduce their impact on other community members indirectly through fear and intimidation (Menge, 1995; Preisser *et al.*, 2005). Consumer effects often dictate the realized niches of prey species (Brooks & Dodson, 1965; Duffy & Hay, 2001; Vonesh *et al.*, 2009), and consumers introduced into new environments may drive some prey to local extinction (Fritts & Rodda, 1998; Mack *et al.*, 2000).

In lentic freshwater habitats, the influences of predation and hydroperiod create a habitat gradient that ranges from small temporal pools to large permanent lakes and reservoirs (Wellborn *et al.*, 1996). Large consumers capable of facilitating top-down trophic cascades—such as fishes—are often absent from ephemeral environments because they are unable to cope with the frequency of drying (Zaret, 1980). As the permanence gradient increases, so does the diversity and density of predatory species (Wellborn *et al.*, 1996; Wilbur, 1997). In the most permanent aquatic habitats, fish are usually present and serve as critical predators, governing the distribution and abundance of many invertebrate and vertebrate prey (Hecnar & McLoskey, 1997; Kurzava & Morin, 1998; Stoks & McPeek, 2003). Fish have been repeatedly cited as imposing a selective sieve that relegates active, undefended, and rapidly developing prey species to ephemeral habitats while selecting for less active, cryptic, and slower developing species in permanent aquatic environments (Zaret, 1980; Werner & McPeek, 1994; Wellborn *et al.*, 1996).

Prey, however, are not passive players in the ecological and evolutionary game; prey species are under intense selection to avoid or deter consumption (Dawkins & Krebs, 1979). This selection manifests itself through the evolution of an array of antipredator adaptations including: morphological traits such as cryptic coloration (Endler, 1990) or spines (Luning, 1992), behavioral adaptations like diel activity changes (Ohman *et al.*, 1983) or habitat shifts (Turner *et al.*, 1999), or chemical defenses such as toxic skin secretions (Berenbaum, 1995) or the ability to detect predator kairomones (Zimmer & Ferrer, 2007). These defenses span a plasticity continuum ranging from obligately constitutive defenses to highly inducible ones (Tollrian & Harvell, 1999). Because of these adaptations to escape or deter predation, many prey species coexist with consumers, resulting in a mosaic of species distributions that vary temporally and spatially with profound effects on population, community, and ecosystemic processes (Hairston *et al.*, 1960; Sih *et al.*, 1985; Duffy & Hay, 2001).

Our knowledge of chemically-mediated prey defenses and their ecological and evolutionary impacts is relatively nascent compared to our understanding of antipredator behaviors and morphologies (Glendinning, 2007; Zimmer & Ferrer, 2007; Meinwald & Eisner, 2008). Chemical ecology is a relatively new field, and most of the attention thus far has focused on plants and their herbivores or on plant-like, sessile marine invertebrates (e.g. tunicates, sponges) and their consumers (e.g. Rosenthal & Berenbaum, 1992; McClintock & Baker, 2001; Hay, 2009). Chemical defenses among higher trophic level species has received less consideration, especially among vertebrates in freshwater systems (Burks & Lodge, 2002).

Among vertebrates, amphibians are probably the most notable and diverse taxonomic class with regard to putative chemical defenses and the diversity of both species and chemistry (Daly *et al.*, 1987; Daly, 1995). Amphibians are distributed on every continent except Antarctica and occupy habitats ranging from deserts to brackish waters (Duellman, 1999; Wells, 2007). Because they are efficient converters of ingested energy into

biomass, amphibians offer a ready source of protein and serve as a critical link between trophic levels (Davic & Welsh, 2004; Wells, 2007). Given that they are small, soft bodied, and lack obvious defenses, it is unsurprising that frogs and salamanders have been called "the cannon fodder of the animal kingdom" (Tyler, 1976). Yet some amphibians secrete a literal pharmacopeia of noxious compounds, including alkaloids, amines, proteins, peptides, and steroids (Daly *et al.*, 1987; Daly, 1995), usually via granular skin glands. As of 2005, over 800 alkaloids had been described from amphibian skin (Daly *et al.*, 2005). These molecules can be neurotoxic, cardiotoxic, haemotoxic, myotoxic, hypotensive, hallucinogenic, anesthetic, or mechanical (e.g., sticky) in action (Toledo & Jared, 1995; Wells, 2007). Dramatic examples of toxic amphibians include the rough-skinned newt (*Taricha granulosa*), a salamander species capable of harboring up to 14 mg of tetrodotoxin (TTX) per individual—enough to kill up to 25,000 mice (Wakely *et al.*, 1966; Hanifin *et al.*, 2008). The skin of *Phyllobates terribilis*, the golden dart frog, can contain up to 1.9 mg of the cardiotoxic and neurotoxic batrachotoxin, one of the most poisonous naturally-produced molecules (Myers *et al.*, 1978; Daly *et al.*, 1980). However, both *T. granulosa* and *P. terribilis* are readily consumed by a few well-adapted predators (Myers *et al.*, 1978; Brodie III & Brodie, 1990).

Despite the plethora of information about amphibian chemical defenses, the relevant predators of most chemically defended amphibians are poorly documented (Daly *et al.*, 1987; Daly, 1995; Toledo & Jared, 1995). This knowledge gap is often masked by the numerous anecdotal and observational accounts of prey unpalatability—and predator resistance—in the literature. As with *Taricha* newts, the eastern newt (*Notophthalmus viridescens*) is commonly assumed to deter predation through the secretion of TTX (Wakely *et al.*, 1966; Levenson & Woodhull, 1979; Yotsu *et al.*, 1990). Indeed, all life-history stages are reportedly unpalatable to a variety of vertebrate and invertebrate predators (e.g. Brodie, 1968; Hurlbert, 1970b). Despite the number of studies involving eastern

newts, there are only a handful of *statistically rigorous* and *ecologically relevant* experiments that confirm the distastefulness of eastern newts to a natural predator. Additionally, rigorous experiments indicating the resistance or tolerance of some predators to eastern newt defenses are rare (but see Brodie, 1981; Hileman *et al.*, 1995). We emphasize statistical rigor because the studies to date did not have adequate replication or controls, some admittedly so (i.e., Hurlbert, 1970b). We also emphasize ecological relevance because, in many other studies of chemical defenses, intraperitoneal injections of newt skin, ova, or purified toxin were used, often to the exclusion of predation bioassays with live prey. Unfortunately, injections remove predator choice from the equation as well as physiological (e.g., salivary proline-rich proteins or cytochrome p450 enzymes) and/or behavioral detoxification methods (e.g.,feeding only on undefended tissues) that might occur during digestion (Glendinning, 2007).

The lack of rigorous research on newt predators and defenses is surprising given that eastern newts have been considered keystone predators that regulate the diversity and abundance of tadpole species, aquatic invertebrate species, and the primary productivity of some temperate freshwater environments (e.g., Morin, 1981, 1983; Wilbur *et al.*, 1983, but see Fauth, 1999). Therefore, we designed a series of experiments empirically testing the hypothesis that *Notophthalmus viridescens* are unpalatable to ecologically relevant generalist predators because of noxious skin secretions. We conducted laboratory-based feeding bioassays where we offered newts to fish predators (i.e. largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*)). We also included crayfish as an invertebrate predator representative of both temporary and permanent lentic environments. Additionally, we tethered adult newts in ponds with and without predatory fish to test the hypothesis that the risk of predation for amphibians, even chemically-defended ones, is higher in permanent ponds containing fish (Wellborn *et al.*, 1996). We then assessed whether earlier life-history stages (embryos and larvae) were more palatable to predators. We also conducted bioassay-guided fractionation to empirically validate the

theory that eastern newt defenses are chemical in nature. Finally, we conducted laboratory feeding trials with naïve young-of-year bluegill sunfish to assess whether and how quickly potential predators could learn to avoid a gradient of relevant TTX concentrations, the putative chemical agent responsible for predator deterrence in eastern newts. Our study hopefully provides a window into the nuances of chemically mediated predator-prey interactions and the importance of ecologically relevant experimentation when addressing the impacts of predation on chemically defended prey.

CHAPTER II

METHODS

2.1 Ethics Statement

All procedures were approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology under permit #A08047.

2.2 Study System & Collections

The eastern newt—*Notophthalmus viridescens* (Rafinesque), Salamandridae—ranges from southern Florida to eastern Texas and north into parts of the Canadian Shield and is one of the most widely distributed salamanders in North America (Petranka, 1998). Sizable newt populations (Gill (1979)) reported $>$ 2000 breeding adults in one pond) are found in a variety of permanent and ephemeral aquatic environments. Shallow wetlands ($\leq$ 1 m) teeming with aquatic vegetation (e.g. *Chara, Utricularia spp.*) are ideal habitats, but large population densities ($\sim$5 adults$\cdot$m^{-2}) are found in lakes, beaver impoundments, grassy marshes, floodplain swamps, vernal pools, and human-modified environments such as farm ponds and roadside ditches (Gates & Thompson, 1982; Harris *et al.*, 1988; Petranka, 1998).

Eastern newts have one of the most complex and variable life histories of any animal (reviewed in Petranka, 1998). Generally, females lay eggs individually in the spring, often wrapping vegetation around each egg for protection. After hatching, the larval period lasts 2–5 months, followed by 1–5 years on land as an eft before returning to an aquatic existence as an adult (Petranka, 1998). Some populations express high frequencies of paedomorphism, in which they sexually mature while retaining larval traits (e.g., gills), while others skip the eft stage and metamorphose directly into sexually mature adults

(Healy, 1973; Gould, 1977).

Like some other Salamandrid species, *N. viridescens* secrete tetrodotoxin (TTX), a neurotoxin that may render the newts unpalatable to predators (Wakely *et al.*, 1966; Levenson & Woodhull, 1979; Yotsu *et al.*, 1990). Red efts have the highest concentrations of TTX, followed by adults, eggs, and finally the larvae (Wakely *et al.*, 1966; Yotsu-Yamashita & Mebs, 2001, 2003). Anecdotal reports note that newts are distasteful and toxic to many predators, including predatory fish, garter snakes (*Thamnophis sirtalis*), and *Ambystoma* salamanders (e.g. Webster, 1960; Brodie, 1968; Hurlbert, 1970a); however, as mentioned above, many of the experimental designs did not employ statistical analyses and lacked both controls and adequate replication.

We collected adult *N. viridescens* by seine and dipnet from Georgia ponds in Walker, Raburn, and Athens-Clarke counties during the summers of 2008 and 2009. Newts were housed in aquaria with dechlorinated tap water at the Georgia Institute of Technology and fed sinking fish pellets and live mealworms *ad libedum*. Newts started mating in captivity in early February. To encourage egg laying, we provided pieces of the aquatic macrophyte *Myriophyllum aquaticum* as a substrate. Each plant was checked daily for freshly laid eggs that were removed to avoid egg cannibalism from other adults. Eggs were maintained individually in $12 \times 12 \times 10$ cm plastic containers containing dechlorinated tap water (6 cm depth), changed biweekly. After hatching, neonate larvae were fed freeze-dried copepods (Cyclop-eeze®) three times a week and provided a small piece of window screen for cover.

We used adult largemouth bass (*Micropterus salmoides*, Centrarchidae) to assess newt palatability to a common predatory fish. Largemouth bass are voracious generalist predators who often consume amphibians across all life-history stages (Page & Burr, 1991; Hecnar & McLoskey, 1997). *Micropterus salmoides* historically ranges from Florida to the Hudson Bay and west to the Mississippi river drainages. The species has been widely introduced throughout North America and is now cosmopolitan; it frequently shares ponds with *N. viridescens* (Page & Burr, 1991; Petranka, 1998). In September 2008, 11

adult largemouth bass (15–20 cm standard length (SL)) were collected via electrofishing from the Chattahoochee National Recreation Area (Morgan Falls Unit near Atlanta, GA, USA) and individually housed in 76 l aquaria. Fish were given a clay flowerpot as refuge and fed earthworms (*Lumbricus terrestris*) every second day.

Bluegill sunfish (*Lepomis macrochirus*, Centrarchidae) were employed as a second fish predator. Like other *Lepomis spp.*, bluegill are generalists that can heavily impact amphibian communities (Smith *et al.*, 1999; Werner & McPeek, 1994). These fish are appropriate model predators because they co-occur with eastern newts, live among dense vegetation where they are likely to encounter *N. viridescens* eggs, larvae, and adults, and their maintenance in the laboratory is relatively tractable. We obtained juvenile (young-of-year (YOY)) bluegill (2–4 cm SL) from the Walton Fish Hatchery (Georgia Dept. of Nat. Resources (DNR), Walton County, GA) in the fall of 2008 and 2009. Juveniles—because they require less food to obtain an observable response—minimized the amount of assay materials needed (i.e. euthanized newts,TTX). Fish were maintained in dechlorinated water in 40 l aquaria that had ben divided in half with plastic mesh. They were fed a maintenance diet of sinking fish pellets (HBH African Cichlid Attack®) every second day.

Procambarus clarkii, the Louisiana red crayfish, is a common invertebrate consumer native to the southeastern United States and northern Mexico (Hobbs, 1989). Commercial exploitation for food and fishing bait has extended the crayfish's distribution worldwide (Lodge *et al.*, 2000). These generalist consumers dramatically alter the structure of freshwater amphibian communities through predation and aggression (Gamradt & Kats, 1996; Gamradt *et al.*, 1997; Lodge *et al.*, 2000). Moreover, because crayfish are not gape-limited, they can prey on adult salamanders as well as eggs and larvae. Adult *P. clarkii* were collected from the Clayton County Water Authority's experimental wetlands in 2008 via minnow traps baited with cat food. They were individually housed in $12\times12\times10$ cm plastic containers within a recirculating freshwater table at the Georgia Institute of Technology. We fed *P. clarkii* a maintenance diet of BIO-blend® herbivore food (Marineland)

2–3 times per week.

We used paedomorphic mole salamanders (*Ambystoma talpoideum*) as palatable controls for the laboratory bioassays. *Ambystoma talpoideum* often co-occur with eastern newts in the Georgia piedmont and coastal plain; paedomorphs are functionally similar to adult *N. viridescens* in terms of swimming ability and size (Petranka, 1998). However, *A. talpoideum* are palatable to many predatory fishes (Semlitsch, 1988) and thus were appropriate controls for this bioassay. The paedomorphs were collected from the University of Georgia's Whitehall Forest in the summers of 2008 and 2009 and maintained identically to eastern newts.

Larval spotted salamanders (*Ambystoma maculatum*) were used as palatable controls for larval bioassays. Like *A. talpoideum*, spotted salamanders are sympatric with eastern newts. While the adults are terrestrial, *A. maculatum* larvae frequently share fishless, usually ephemeral, ponds with both larval and adult eastern newts. The females of spotted salamanders deposit egg masses (1–250 eggs per mass) in the late winter and early spring that hatch after 4–7 wks of incubation (Petranka, 1998). In March 2010, we collected two egg masses from a spring-fed pool on a private farm in Palmetto, GA. The eggs and hatched larvae were housed at Georgia Tech in a 40 l aquarium with dechlorinated tap water. Larvae were fed freeze-dried copepods three times per week, then slowly graduated to sinking fish pellets as they grew larger.

2.3 Laboratory feeding assays

2.3.1 Largemouth bass

We simultaneously offered an adult *N. viridescens* and a paedomorphic *A. talpoideum* (as a palatable control) to largemouth bass (n = 11) in a paired-choice predation bioassay. Prior to the experiment, bass were fed one large earthworm to ensure feeding choices did not reflect starvation. The bioassay initially lasted 14 h but was continued for 72 h to observe whether hunger would overwhelm any distastefulness. At the experiment's

conclusion, each replicate tank was thoroughly inspected and the remaining prey species were noted. We used a two-tailed McNemar test for paired samples to detect differences in the numbers of each salamander species consumed. All statistical analyses were run in *R* (R Development Core Team, 2009) except where noted.

2.3.2 Bluegill

We used young-of-year bluegill as predators for assessing the palatability of differing newt body tissues. In fall 2008 three adult newts were euthanized by decapitation. Once euthanized, they were skinned, taking care to keep the dorsal and ventral skin separate; the internal organs were then removed. The three newt tissues (i.e. dorsal and ventral skin, viscera) were subsequently cut into small pieces approximately 5 mm^2 and pooled for the bioassay.

Individual bluegill were first offered a control food pellet (HBH Cichlid Attack®). If they accepted, they were offered one of the three treatment tissues (n = 10 per treatment). If the treatment food was rejected, fish were offered a second control pellet to ensure the rejection response was not due to satiation. One fish receiving the ventral skin treatment would not accept a second food pellet and was excluded. In early 2010 the experiment was repeated; there was no significant date effect (P = 0.49) between the two bioassays so we combined the data (dorsal skin: n = 15, ventral skin: n = 17, viscera: n = 16). The percent acceptance of each treatment relative to the acceptance of the first control pellet was first analyzed separately with a two-tailed McNemar test. The relative palatability across newt tissue treatments was determined with a generalized linear model with a binomial error distribution in *SPSS* (v. 18.0). We conducted post-hoc pairwise comparisons using Fisher's LSD following Bonferroni correction (α = 0.016).

2.3.3 Crayfish

We evaluated whether eastern newts were distasteful to adult *P. clarkii* (n = 10) in a paired-choice feeding assay. Crayfish were acclimated for 48 h in glass aquaria (51$\times$27 cm)

containing dechlorinated tap water at a depth of 6 cm. Immediately prior to the bioassay, 10 newts and 10 *A. talpoideum* were euthanized with a lethal dose of MS-222 (tricane methylsulfonate, Finquel) followed by a thorough rinse with deionized water. This minimized prolonged prey suffering and removed any differential effects of escape behavior between these potential prey, allowing a test of palatability alone. We simultaneously offered crayfish one newt and one *A. talpoideum*; after 18 h, both prey choices were removed and percentage eaten assessed. Because crayfish are messy eaters, a prey item was considered consumed if more than 50% of the individual was eaten. We quantified differences in the consumption of both salamander species with a two-tailed McNemar test.

2.3.4 Newt palatability as a function of ontogeny

Bluegill (n = 10) were offered eggs of *N. viridescens* alongside a sinking food pellet control to ascertain whether the eggs are distasteful to a common predatory fish. The paired-choice feeding assay was identical to the assay described for bluegill above (Section 2.3.2). Bluegill were first offered a food pellet followed by a newt egg. If the egg was rejected, a second control pellet was offered to assess whether that fish was satiated. We considered an egg rejected if the fish struck the egg, spit it out, and then ignored it. We used a McNemar test to compare the number of initial control pellets and newt eggs eaten.

To determine whether newt hatchlings are distasteful to fish, we offered bluegill a neonate newt and an *A. maculatum* hatchling in a paired-choice assay (n = 13). We randomized the order of treatment that each fish received to normalize effects of feeding order. Spotted salamander neonates are slightly larger than *N. viridescens* hatchlings (12–17 mm and 7–9 mm respectively; Petranka, 1998), but juvenile bluegill showed no size-based attack preference and pursued both larval species equally. Because the newts did not hatch simultaneously, the bioassay was staggered over two days, ensuring that

no newt larva was over 1 d old. No bluegill individual was used more than once. We determined whether there were differences in bluegill preference with a two-tailed McNemar test.

2.4 Field predation experiment

Table 1. Summary and locational information of the six pond pairs used to assess the relative predation of tethered *N. viridescens* in ponds with and without fish.

Pond	Location	County/State	Predatory Fish	*n*
Silty Pond Dove Pond	Charlie Elliot WMA	Newton, GA	Fishless Bass, *Lepomis*, Catfish	10
Coverboard Pond Three Sisters Lake	Whitehall Forest	Clark, GA	Fishless Bass, *Lepomis*, Catfish	10
Snake Lake Sawmill Lake	Walker-Pigeon Mt.	Walker, GA	Fishless Bass, *Lepomis*, Catfish	12
N/A Dean Pond	Whitehall Forest	Clark, GA	Fishless Bass, *Lepomis*, Catfish	11
N/A N/A	Stallings Farm	Knox, TN	Fishless Bass, *Lepomis*	10
Wetland Unit Shamrock Lake	Clayton Co. Water Authority	Clayton, GA	Fishless Bass, *Lepomis*, Catfish	9

We quantified the relative risk of predation for eastern newts in habitats with and without fish by tethering adult *N. viridescens* in paired, permanent ponds (n = 6 paired ponds with 9–12 newts per pond) for 18 hrs in fall 2009—one pond per pair contained predatory fish (e.g. largemouth bass, *Lepomis spp.*, catfish (*Ictalurus spp.*) and one pond per pair lacked fish (Table 1). We chose to use permanent ponds because (1) *N. viridescens* often reach their greatest abundances in permanent fishless ponds (Petranka, 1998) and so are relevant for comparison of predation regimes, (2) to minimize any differences associated with the abiotic impact of drying (e.g., salinity, conductance, dissolved O^2 levels), and (3) the southeastern US was recovering from the effects of a severe drought and there were few ephemeral ponds still holding water. Ponds were located no more than 2 km apart, both making the experiment tractable and minimizing geographical differences. All

ponds were drag-seined and dipnetted to identify the fish species present. Additionally, many fish-containing ponds were stocked for public fishing by the Georgia DNR so species presence could be verified with DNR catalogs of fish presence. Two ponds we categorized as fishless (Stalling Farm and Clayton County Water Authority's wetland pond) did contain mosquitofish (*Gambusia holbrooki*). *Gambusia spp.* are known to feed on newt larvae (Gamradt & Kats, 1996) but are too small to threaten adults and so were not considered predatory.

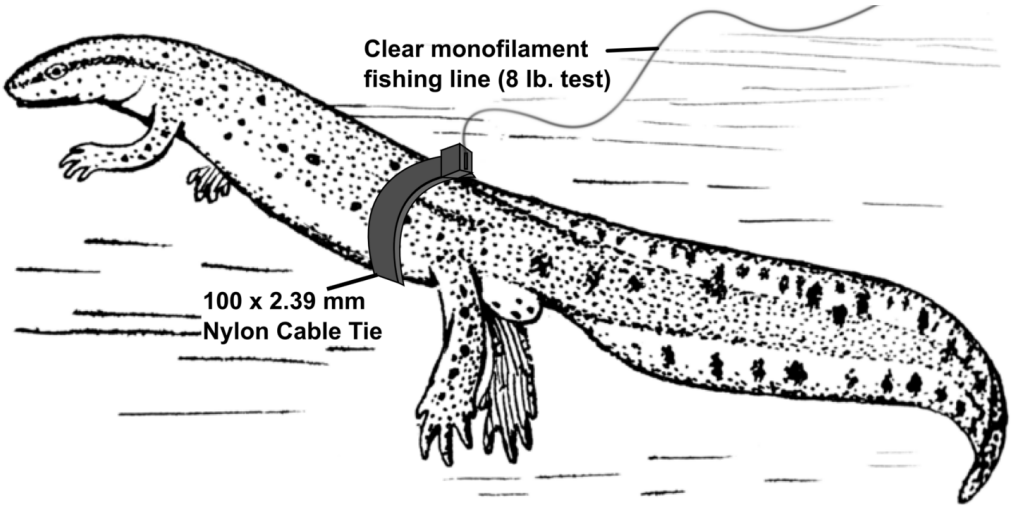

Figure 1. Schematic illustrating how eastern newts were tethered for the field predation experiment.

Newts were tethered by fastening a nylon cable tie (100×2.39 mm) around the torso just anterior of the pelvic girdle (Figure 1). Prior to securing the cable tie, we fastened approximately 3 m of clear 3.63 kg test monofilament fishing line through the tie's ratchet case. The cable tie was tightened until the newt could not slip out but was left loose enough to rotate freely and allow unconstrained movement. The remaining length of cable tie was then cut flush with the ratchet case. We secured the fishing line to the pond bank with a metal stake (22.86 cm). The newt was then placed at the water's edge and allowed to enter on its own.

Newts were spaced no closer than 6 m apart and we monitored their presence or

absence every two hours. We recorded newt presence, survival, and injuries from failed predation attempts. Potential predators near each individual were also noted (e.g. Ranid frogs, turtles, predacious invertebrates). We calculated the percent survival of newts in each pond and analyzed the data with a paired t-test, using pond pair as the level of replication.

2.5 Bioassay-guided fractionation of newt extract

2.5.1 Artificial food methods

Because adult *N. viridescens* were consistently rejected by predators, we used bioassay-guided fractionation to assess whether rejection was (1) due to chemical deterrents and (2) could be explained by the presence of TTX. To determine effects of newt chemistry, we incorporated *N. viridescens* extracts into an gel-based artificial food constructed with freeze-dried and finely ground frog legs, sodium alginate (27% by dry mass), and 25 μg of red food coloring for visibility (see Hay *et al.*, 1998, for a general overview) and fed these treatment foods vs. identical control foods (but without the newt extract) to juvenile bluegill. The gel was loaded into a small syringe, squeezed into lines on a glass petri dish, and misted with a hardening solution of 0.25 M calcium chloride. After $\sim$1 min, the gel was cut into bite-sized pellets. This method resulted in 'noodles' with the consistency of cooked pasta. Extracts were tested at double their extracted concentrations (by volume) to offset loss due to inefficient extractions or losses because of chemical decomposition (for an example of such losses, see Parker *et al.*, 2006).

2.5.2 Feeding assays

Juvenile bluegill were acclimated in divided aquaria as described in Section 2.2. Bluegill received two food pellets every morning prior to the bioassays to ensure feeding responses were not starvation-induced. In the afternoon fish individuals—assigned to treatments at random and interspersed spatially—received either a pellet containing newt extract or a control pellet and were monitored for acceptance or rejection of the treatment.

No fish was used more than once. We determined statistical differences between experimental extract treatments and control treatments with a two-tailed Fisher's exact test.

2.5.3 Extraction methods

Extracts of *N. viridescens* were acquired by euthanizing and eviscerating 10 adult newts. We gutted the newts because secretion of chemical defenses in amphibians occurs primarily via dermal granular glands (Toledo & Jared, 1995) and because our previous feeding assays indicated a rapid rejection that suggested metabolites are presented on newt exteriors; thus we focused on the skin. The newt tissue was macerated in a blender with methanol and water (7:3 v:v) and extracted overnight, then successively extracted for at least two hours in methanol and dichloromethane (1:0 v:v, 1:1 v:v, and 0:1 v:v). Solvents were subsequently removed under vacuum by rotary evaporation and the extract was then resuspended in methanol for bioassays. For the artificial food, the extract was incorporated into the frog-leg powder and sodium alginate mixture, evaporated under vacuum in a speed-vac to remove the solvent, and prepared as described above. The method for control foods was identical, including the solvent, but lacked the newt extract.

Following confirmation of deterrent activity, the remaining crude extract was partitioned into lipophilic and polar fractions with ethyl acetate and distilled water; both fractions were tested separately for deterrent activity using the methods described above (Sections 2.5.1–2.5.2) except that the polar fraction was resuspended in distilled and deionized water.

2.6 Bluegill avoidance conditioning

We wanted to evaluate how quickly naïve predatory fish would learn to avoid food containing TTX concentrations commonly found in eastern newts. Whole animal concentrations of TTX reported for *N. viridescens* range from < 0.15–24 μg·g^{-1} (Yotsu-Yamashita & Mebs, 2001) to 146 ± 6 μg·g^{-1} of wet mass (Yotsu *et al.*, 1990). In 2008 we reconstituted

20 μg·g^{-1} of tetrodotoxin citrate (Ascent Scientific) into a gel-based artificial diet after re-suspension in distilled and deionized water. This TTX concentration is on the low end of the range for eastern newts but slightly higher than the mean concentration reported for a population in Highlands, North Carolina (Yotsu-Yamashita & Mebs, 2001). The control pellets were constructed identically but lacked TTX.

We acclimated 60 bluegill in 30 divided aquaria (one fish per side) as described in Section 2.2. Each morning they received two food pellets to ensure feeding responses were not starvation-induced. In the afternoon an individual received either a TTX-containing frog pellet or a control pellet. Fish were assigned to treatments by random interspersion. Over 4 d, we monitored whether fish ate the offered pellet or rejected it. Because some treatment fish died, survival was also noted. One fish receiving TTX pellets never fed and was excluded from the analysis. The data were analyzed with a generalized linear mixed model (GLMM) fit by Gaussian Hermite approximation (nAGQ = 15) and binomially distributed errors using *R*'s *lme4* package (Bates & Maechler, 2009). We started with a saturated model and used Akaike's Information Criterion (AIC; Akaike, 1973) to remove all terms but the treatment $\times$ day interaction as a fixed factor and fish ID as a random factor. We evaluated significance with the Wald F statistic on 56 residual degrees of freedom. Determining statistical significance in GLMM is difficult because of uncertainty regarding the appropriate number of denominator degrees of freedom. However, by using the individual fish as our unit of replication, we minimized type I error.

In 2009, we repeated the avoidance conditioning experiment, but expanded the study to investigate bluegill responses to quantitative differences in TTX concentration. Over 6 d and five treatments, we exposed naïve bluegill to a gradient of TTX concentrations: 0, 10, 20, 40, and 80 μg·g^{-1} of TTX (n = 20·treatment^{-1}). The highest concentration (80 μg·g^{-1} TTX) is 55% of the highest concentration reported for *N. viridescens* (146 $\pm$ 6 μg·g^{-1} Yotsu *et al.*, 1990). Other than the addition of treatments, the methodology was identical. One fish in the 10 μg·g^{-1} treatment and two fish in the 80 μg·g^{-1} treatment refused to

feed throughout the experiment and were excluded from the analysis. As with the previous experiment, we analyzed the data via a GLMM with binomially distributed errors using the *lme4* package (Bates & Maechler, 2009). We used AIC to select the model with treatment and day as fixed factors and fish ID as a random factor on 92 residual degrees of freedom and Tukey HSD for multiple comparisons (*multcomp* package; Hothorn *et al.*, 2008).

CHAPTER III

RESULTS

3.1 Are adult newts unpalatable in the laboratory?

Largemouth bass (n = 11) consumed 10 paedomorphic mole salamanders but only one newt in a paired-choice assay (Figure 2; P = 0.004). Bass took newts into their buccal cavity and immediately spit them out, followed by coughing and rapid operculation. A few bass repeatedly attacked the newts only to reject them each time. Two newts had abrasions that suggested they had been chewed in the pharyngeal jaws before rejection, but the injuries were not fatal. In contrast, no bass ever rejected *A. talpoideum*.

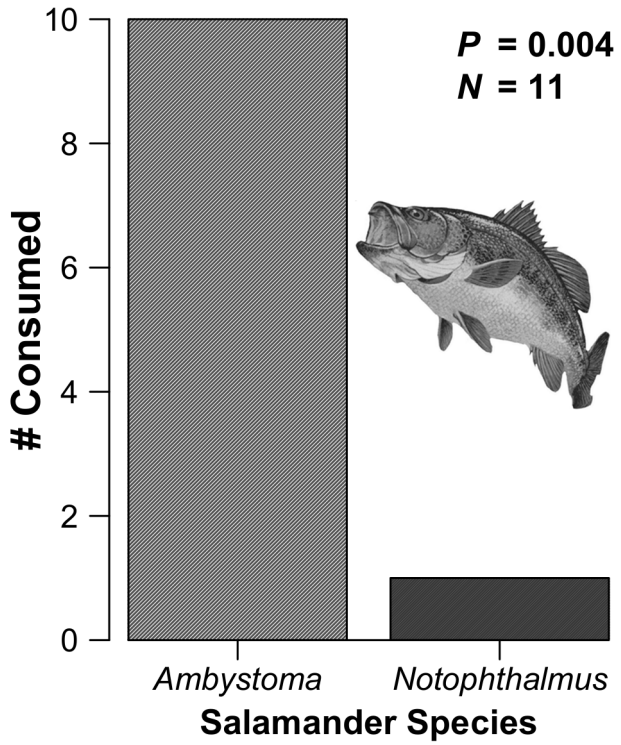

Figure 2. Largemouth bass consumption of salamanders in a paired-choice predation assay. Bass consumed significantly more control salamanders (paedomorphic *Ambystoma talpoideum*) than eastern newts, indicating newts are unpalatable to this predator (McNemar test: P = 0.004, n = 11).

Juvenile bluegill consumed significantly less dorsal and ventral skin tissue of eastern newts relative to control pellets in a paired-choice feeding assay (Figure 3). Fish ate 100% of the controls but only 20% of the dorsal skin and 35% of the ventral skin offered ($P \leq$ 0.003 for both treatments vs. the control). Fish consumed 75% of the newt viscera ; this did not differ significantly from control pellets (P = 0.073, n = 16). A GLM indicated significant palatability differences among the three tissue treatments (P = 0.016, df = 2, 46). Pairwise comparisons after Bonferroni correction (α = 0.016) showed dorsal skin was significantly less palatable than viscera (P = 0.01); palatability of ventral skin fell intermediate between viscera and dorsal skin and did not differ significantly from either. There was, however, a trend for viscera to be more palatable than ventral skin (P = 0.06).

The generalist crayfish *P. clarkii* ate paedomorphic *A. talpoideum* in preference to eastern newts in a 9:1 ratio, although many of the newts exhibited missing toes and tail tips (Figure 4; P = 0.008, n = 10). Moreover, the single crayfish that ate a newt had eviscerated the newt and consumed the internal organs, legs, and tail, leaving the dorsal skin rejected and intact.

3.2 How does palatability change with ontogeny?

Only 30% of the bluegill consumed the eggs of *N. viridescens* compared to 100% acceptance of controls in a paired-choice predation bioassay (Figure 5; P = 0.023, n = 10). All of the fish that rejected eggs subsequently coughed and operculated rapidly. Four of seven rejected eggs were still viable following the rejection and hatched soon after.

When offered newt hatchlings and larvae of spotted salamanders, bluegill consumed 100% of the spotted salamanders and 62% of the newts. This difference was not significant in a two-tailed test (P = 0.073, n = 13) but is suggestive. The five fish that rejected newt hatchlings took up to 2 min before spitting the larvae out. None of the rejected larvae survived.

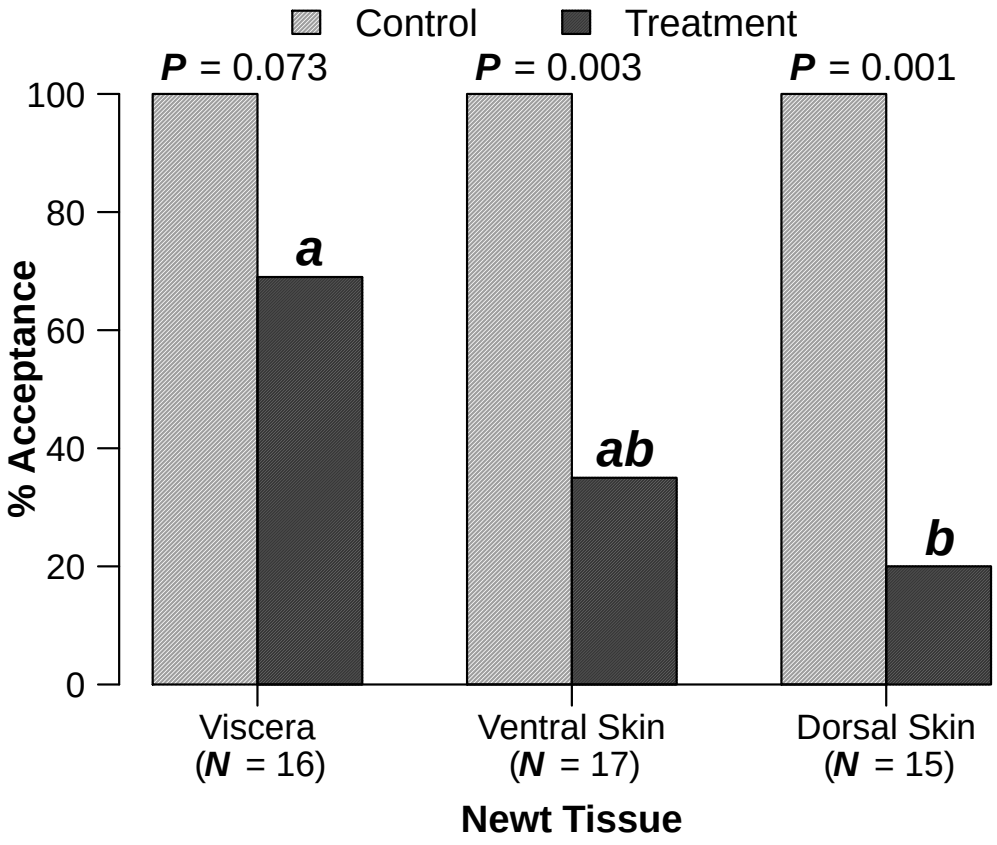

Figure 3. Fish consumption of three different *N. viridescens* body tissues. Bluegill (*Lepomis macrochirus*) consumption of newt viscera and control pellets did not differ statistically (McNemar Test: $P = 0.073$, $n = 16$). However, fish found the ventral and dorsal skin significantly more distasteful than control pellets (ventral skin: $P = 0.003$, $n = 17$; dorsal skin: $P = 0.001$, $n = 15$). Overall, newt tissues statistically differed from each other in palatability (GLM: Wald $\chi^2_{2,46} = 8.241$, $P = 0.016$, $df = 2,46$); bars that share the same letter are not statistically different from each other after Bonferroni correction ($P < 0.016$).

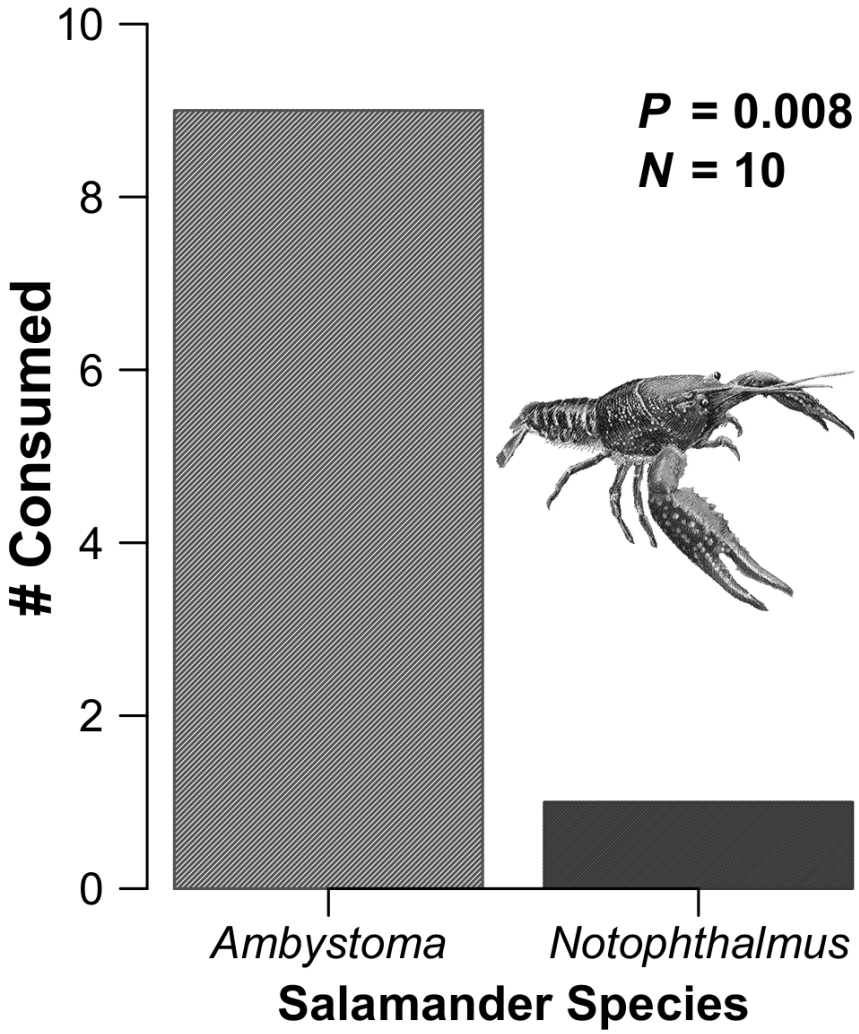

Figure 4. Consumption of salamanders by crayfish in a paired-choice predation assay. Crayfish (*Procambarus clarkii*) ate significantly more *A. talpoideum* paedomorphs than newts (McNemar test: $P = 0.008$, $n = 10$).

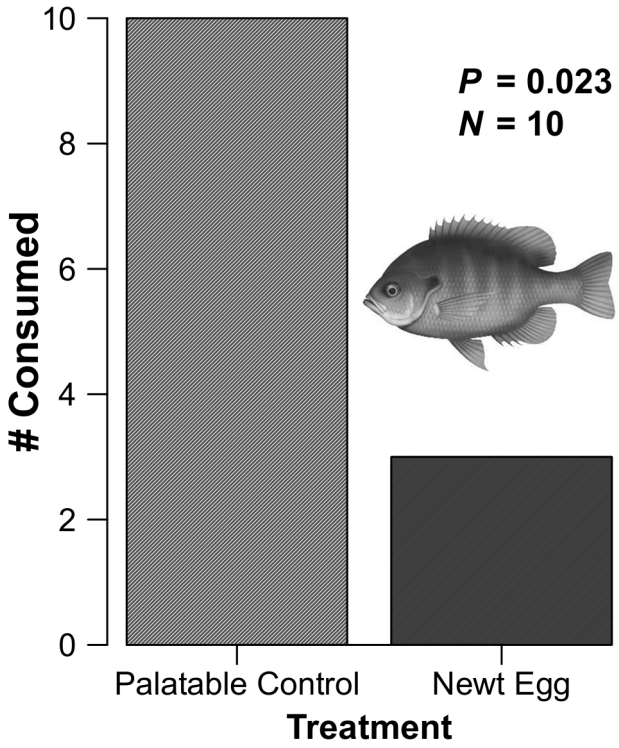

Figure 5. Paired-choice feeding assay with juvenile bluegill as a generalist egg predator. Bluegill ate significantly less eastern newt eggs than palatable control pellet of equal size (Mcnemar test: $P = 0.023$, $n = 10$).

3.3 Are adult newts consumed in the field?

To assess whether *N. viridescens* experienced more predation in ponds containing fish, we tethered adult newts (Figure 1) in spatially paired ponds with and without predatory fish (Figure 6; $n = 6$ pond pairs). After 18 h, newt mortality in ponds with fishes was 55% higher than in ponds lacking fishes (62% vs. 40% respectively; $P = 0.029$). Many victims of predation had various body parts cleanly bitten off. It was not uncommon, upon finding a newt bitten in half, to observe small turtles (Kinosternidae; approx. 5–8 cm) moving away underwater. Moreover, on several occasions bullfrogs (*Rana catesbeiana*) had eaten the newts, regurgitating them as we pulled on the monofilament line to retrieve the newt. One bullfrog was partially paralyzed following newt consumption. The frog was gasping and unable to right itself.

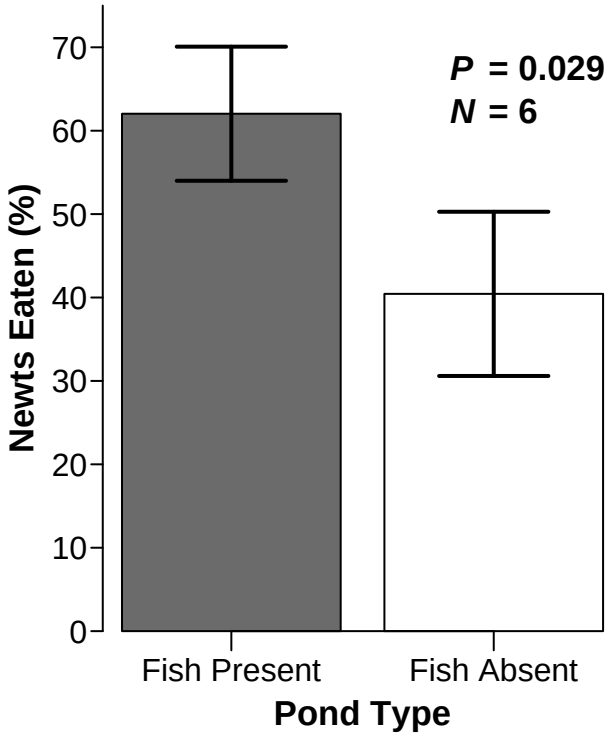

Figure 6. Relative predation frequencies for newts in ponds containing and ponds lacking predatory fish. Predation on *N. viridescens* was significantly higher in ponds containing fish than in ponds lacking fish (paired t-test: $t = 3.031$, $P = 0.029$, $n = 6$). Error bars are mean $\pm$ SE.

3.4 Is newt palatability chemically mediated?

The crude extract of adult eastern newts significantly deterred feeding by bluegill relative to controls (Figure 7; $P = 0.007$, $n = 16$). After separation of the crude extract via liquid-liquid partitioning, activity remained in the water-soluble fraction ($P = 0.018$, $n = 17$). The lipophilic (ethyl acetate) extract did not inhibit feeding by bluegill ($P = 0.487$, $n = 18$).

3.5 Do generalist predators require a learning period for TTX avoidance?

Naïve bluegill learned to avoid artificial food pellets containing ecologically relevant concentrations of TTX (20 $\mu g \cdot g^{-1}$) after 2 feeding events (Figure 8). Over 4 d, 50% of the fish receiving TTX pellets ceased feeding while the proportion accepting control pellets

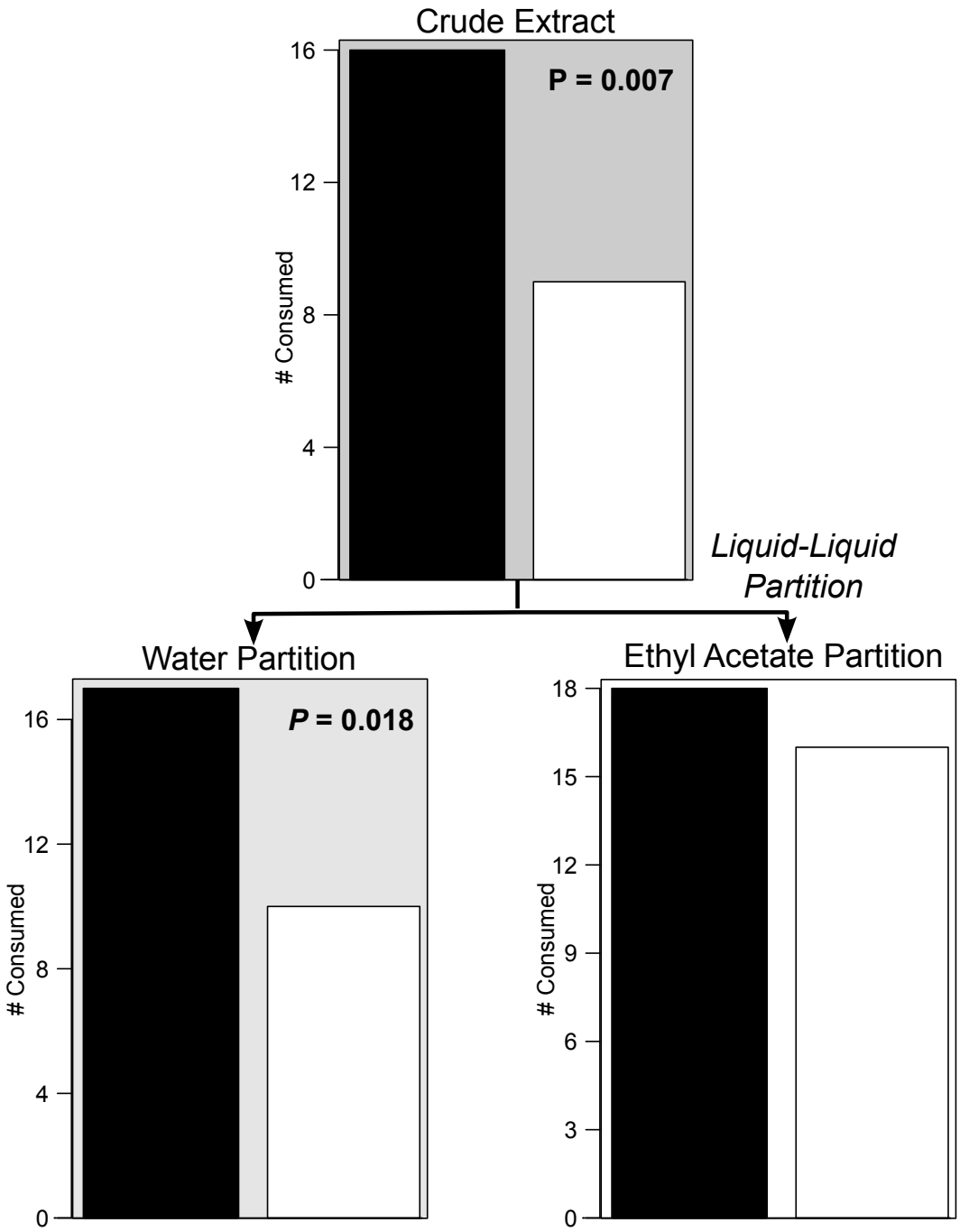

Figure 7. Bioassay-guided fractionation of chemical extracts from *Notophthalmus viridescens*. Each graph shows the number of bluegill feeding on a solvent-only control food (black bar) vs. food containing eastern newt extracts (open bar). Shaded graph panels denote statistically significant ($P < 0.02$) reductions in feeding relative to the palatable control (Fisher's exact tests). See Section 2.5 for chromatographic details.

remained stable, resulting in a significant treatment $\times$ day interaction (GLMM: $P < 0.001$, *df* = 2, 56).

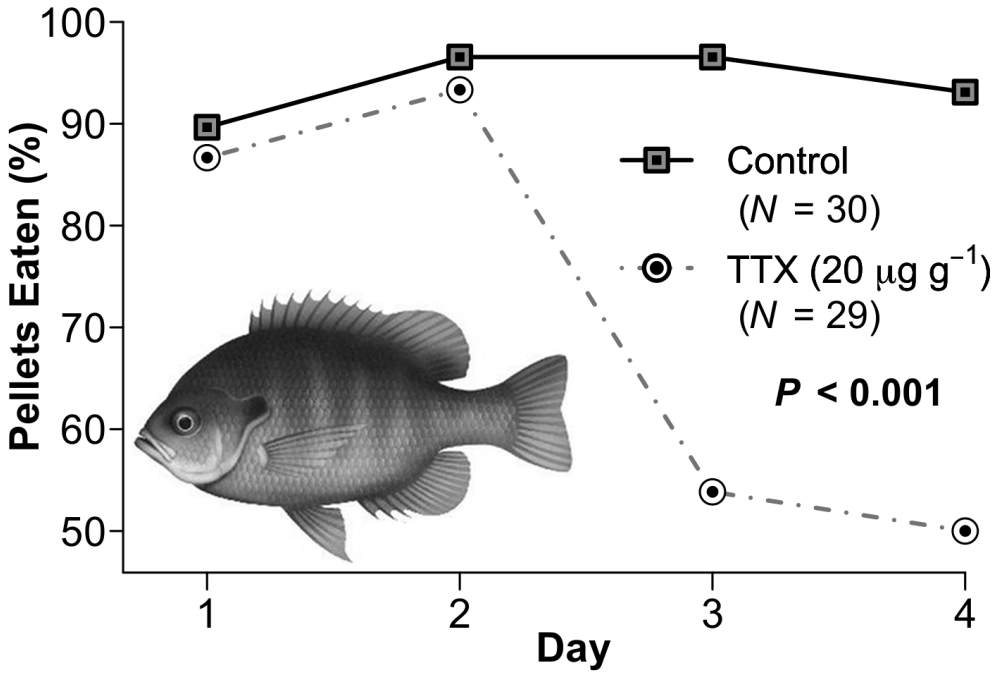

Figure 8. Bluegill consumption rates (% eaten) of pellets containing or lacking TTX over 4 d. Juvenile *L. macrochirus* were offered an artificial food pellet containing ecologically relevant TTX concentrations (20 μg·g^{-1} wet weight) or a sham control. A significant treatment $\times$ day interaction indicates naïve bluegill quickly learn avoidance of food containing TTX concentrations found in eastern newts (GLMM: Wald $F_{2,56}$ = 60.46, $P < 0.001$).

Because the concentrations of TTX reported for *N. viridescens* in the literature vary from $<$ 0.15 μg·g^{-1} (Yotsu-Yamashita & Mebs, 2001) to 148 $\pm$ 6 μg·g^{-1} (Yotsu *et al.*, 1990), we quantified how feeding by naïve fish changed with increasing TTX concentrations (Figure 9). Both TTX treatment and day had significant effects on bluegill consumption ($P < 0.001$, *df* = 4,92; day: P = 0.007, *df* = 1,92). Moreover, Tukey's HSD for multiple comparisons indicated control pellets (0 μg·g^{-1}) were accepted significantly more than any of the four TTX treatments. After 6 d of feeding exposure, consumption of control pellets was 95%, acceptance of pellets with 10 or 20 μg·g^{-1} TTX was $\sim$25%, and acceptance of pellets containing 40 or 80 μg·g^{-1} TTX was only 0–5%. Treatments clustered

into three significant groupings; the highest two concentrations were almost completely rejected, the 10 and 20 μg·g^{-1} concentrations were consumed by a quarter of the fish, and control pellets were eaten by almost all of the bluegill.

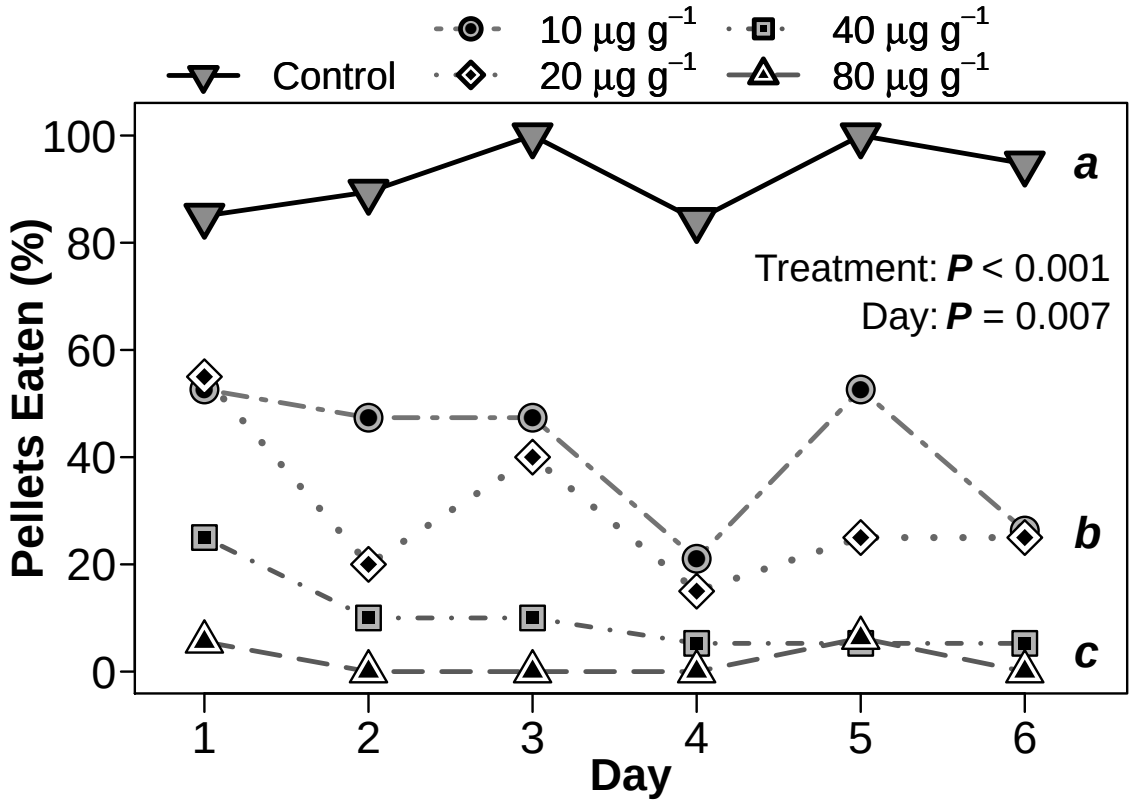

Figure 9. Percent of food pellets eaten by bluegill over 6 d when exposed to a gradient in TTX. Juvenile bluegill were offered artificial food pellets with TTX concentrations ranging from 0–80 μg·g^{-1}. There was a significant effect of both TTX concentration (Wald $F_{4,92}$ = 92.075, $P < 0.001$) and day (Wald $F_{1,92}$ = 7.53, P = 0.007). Treatments sharing the same letter did not differ statistically according to Tukey's HSD.

CHAPTER IV

DISCUSSION

4.1 Adult newts are unpalatable to generalist predators . . . mostly

The significance of consumer impacts on the distributions and abundances of organisms lower on the trophic ladder is well known (Paine, 1966; Murdoch *et al*., 2003), especially for the amphibians inhabiting many lentic freshwater systems (Morin, 1983; Duellman & Trueb, 1994; Wells, 2007). Despite their small soft bodies and high nutritional value, many species of frogs, toads, and salamanders thrive in the face of predation,some by wielding noxious chemical compounds against consumers (Wells, 2007). Yet, outside of a few notable cases, we still know little about the ecological efficacy of these compounds in deterring common generalist consumers. Here we describe the deterrence of adult *Notophthalmus viridescens* and their eggs (Figure 5) to relevant, cosmopolitan consumers (Figures 2–4). We combined analytical chemistry methods with predation bioassays in the laboratory and field to show that eastern newts—despite possessing a water-soluble, skin-based chemical defense (Figure 7) that discourages feeding by generalist fishes—are nonetheless acceptable dietary items to some consumers in their natural environment, especially in fish-containing ponds (Figure 6) .

4.1.1 In the laboratory, newts are resistant to predators in the presence of alternative prey.

The predators used in our bioassays (i.e., largemouth bass, bluegill, *P. clarkii* crayfish) consume a variety of organisms but still selectively feed based prey traits. All three consumers demonstrated selective feeding by rejecting eastern newts (or their dermal tissues) as prey when provided a palatable alternative (Figures 2–4). When offered a prey choice of an eastern newt and a mole salamander, largemouth basses overwhelmingly

consumed mole salamanders while actively rejecting eastern newts, indicating they find adult *N. viridescens* distasteful (Figure 2). Adult newts were also relatively distasteful to the crayfish *Procambarus clarkii*; crayfish were nine times more likely to feed on mole salamander paedomorphs than eastern newts (Figure 4). Our laboratory-based bioassays demonstrate that eastern newts are relatively unpalatable to generalist predators like crayfishes and Centrarchid fishes.

Additionally, bluegill found different pieces of *N. viridescens* differentially distasteful; the viscera were most palatable, the dorsal skin was least palatable, and the ventral skin was intermediate in palatability (Figure 3). Hanifin *et al.* (2004) found a similar gradient in the distribution of both TTX toxicity and the granular glands that secrete TTX in the skin of *Taricha granulosa* newts. However, because differential bluegill consumption of the newt viscera compared to the controls showed a statistical trend towards significance ($P = 0.073$), we are uncomfortable definitively asserting that the viscera are undefended. Rather, there are a few possibilities for the observed result. (1) The viscera may truly lack defenses and may not need them given their enclosure within distasteful skin. Undefended organs also minimize the danger of autotoxicity as well. (2) The viscera may be undefended but became contaminated by unpalatable skin secretions during dissection. (3) The viscera may have some deterrent properties but not enough for reliable detection in this assay. Wakely *et al.* (1966) detected TTX in the ovaries and lower amounts in the blood and viscera of *Taricha granulosa* newts and Yotsu *et al.* (1990) detected similar TTX distributions in the Japanese newt *Cynops eniscauda*, but neither study evaluated the palatability of these tissues to predators.

4.1.2 Despite deterring feeding by laboratory fishes, *N. viridescens* are more susceptible to predation in ponds with fish predators than those lacking fishes.

Given the low preference of newts to common and ecologically important predatory fishes, we expected that consumption of newts in the field might be low. Contrary to our predictions, newts experienced predation in both pond types but especially in ponds containing

predatory fishes such as bass and *Ictalurus* catfish (Figure 6), where mortality was significantly higher. It is uncertain why *N. viridescens* was unpalatable in the laboratory but experienced high mortality in fish-containing ponds. However, possible explanations include: (1) we did not test some critical predators of *N. viridescens*, (2) constraints from tethering increased newt susceptibility to consumption, or (3) our laboratory predation assays did not test absolute preferences of the consumers.

The first explanation for the disconnect between our laboratory and field results is that our laboratory assays did not encompass all relevant predators of newt adults and that newts are consumed by some subset of the diverse assemblage of consumers that occur in the field. The identity of relevant predators remains untested, but there is reasonable evidence to suspect that adult bullfrogs and turtles occasionally consume *N. viridescens*. Bullfrogs grow large enough to eat adult newts as their diet includes snakes, small mammals, fish, and other amphibians (Conant & Collins, 1998; Lannoo, 2005). During newt retrieval we recovered several partially-digested newts from bullfrog stomachs. Brodie (1968) found that bullfrogs could stomach eastern newts and their efts without distress, and Hurlbert (1970a) kept a large bullfrog for months on a diet of *N. viridescens*. Small turtles, most likely musk turtles (*Sternotherus spp.*) or mud turtles (*Kinosternon spp.* were witnessed swimming away from dead newts. Although direct consumption was never observed, the feeding damage was congruous with turtles' shearing beaks. Hurlbert (1970a) also found that snapping turtles (*Chelydra serpentina*) and painted turtles *Chrysemys picta*) would consume *N. viridescens*. However, both the Brodie (1968) and Hurlbert (1970a) studies were observational and lacked replication. Bullfrogs and turtles are predators deserving further investigation.

Second, tethering newts undoubtedly constrains their movement, possibly interfering with consumer evasion (Figure 1). It is possible that distastefulness causes initial hesitation by many consumers and facilitates newt escapes into refuges. Despite this potential artifact, our tethering experiment demonstrated among-habitat differences in mortality

(Figure 6) and provides an initial 'first' effort upon which others can improve. Furthermore, it would be interesting to critically examine the interaction between chemical defenses and behavioral escape responses. Another potential problem was that we had to infer predation from presence/absence data, although in a few instances, we know that frogs consumed newts because we pulled these from their stomachs as we retrieved the monofilament line. Several newts were discovered dead with pronounced skin abrasions that were almost identical to those of newts rejected by largemouth bass during the laboratory predation assays. Accordingly, some newt absences may be a function of failed predation attempts where newts were removed, found unpalatable, and rejected away from the tether.

Third, multiple choice assays are only able to explicitly assess prey palatability *relative* to alternative food items. Yet, for Centrarchid fishes like largemouth bass and bluegill sunfish, we are confident they are unlikely to consume adult eastern newts unless faced with starvation. After 60 hrs without alternative prey, the bass still refused to consume the available newts (Figure 2), and all 11 fish immediately ate two large earthworms following the experiment, suggesting satiation was not an issue. Furthermore, in a newt-rich New York lake, nary a newt was noted in the gut contents of several thousand stomaches from a diverse group of predatory fishes—including smallmouth bass (*Micropterus dolomieu*) and pumpkinseed sunfish (*Lepomis gibbosus*) (George *et al.*, 1977). The propensity for crayfish like *P. clarkii* to consume eastern newts is less clear. Bass and bluegill feed by sucking their prey entire into the oral cavity; however, crayfishes can selectively feed from different body tissues. Indeed, the single crayfish that consumed a newt in our assay (Figure 4) eviscerated it and ate the internal organs, feet and legs; it did not consume the dorsum. There is also evidence that crayfish may have some resistance to tetrodotoxin. In California *P. clarkii* is an invasive predator of *Taricha* newt eggs (Gamradt & Kats, 1996) despite the presence of significant TTX concentrations (Wakely *et al.*, 1966; Hanifin *et al.*, 2003). However, whether crayfish consume adult *Taricha* is unknown.

4.2 Notophthalmus viridescens *is partially defended throughout ontogeny*

The fertilized eggs of *N. viridescens* are unpalatable to generalist fishes such as bluegill, 70% of fish rejected these eggs (Figure 5). Of those that found the eggs distasteful, four fish made multiple attempts at egg consumption before giving up, and the rest immediately rejected the egg after sampling. The three fish that consumed the eggs took considerable time to do so, and their repeated coughing and rapid operculation suggested that they were either in some distress or were attempting to wash the eggs prior to consumption. Largemouth bass displayed similar behaviors in our bioassays with adult *N. viridescens* and in a previous study on whirligig beetles (*Dineutes hornii*)—an insect species defended by the secondary metabolite gyrinidal, a norsesquiterpene (Eisner & Aneshansley, 2000).

Chemical defenses are the most probable mechanism behind the antipredator protection of the eggs, but an attempt to extract individual eggs suffered from methodological problems, and the extracts showed no deterrent activity (*data not shown*); thus we can not discount the presence of mechanical defenses (e.g., tough jelly coat). However, the endowment of eggs or larvae with chemical defenses is not uncommon in nature. For example, pipevine swallowtail females (*Battus philenor*) coat their eggs with toxic aristolochic acids during oviposition; upon hatching, larvae immediately eat the shell, likely to sequester the toxins (Fordyce *et al.*, 2005). In amphibians, Bufonid toad females imbibe their eggs with cardiotoxic bufodienolides (Licht, 1968); *Taricha* newt eggs contain TTX, often in appreciable amounts (Hanifin *et al.*, 2003), and many marine invertebrates produce eggs or larvae containing chemical defenses against consumers (Lindquist & Hay, 1996). Brodie *et al.* (1974) found that macerated ova of *N. viridescens* was fatal to mice when injected intraperitoneally, though the ecological relevance of method is suspect. Because male newts use a spermatophore to transfer sperm (Petranka, 1998), it would be interesting to ask whether males contribute deterrent secondary metabolites as nuptial gifts, and if so, whether females preferentially select the males that provide them with the

most toxic spermatophores. Although unknown in amphibians, some male insects donate secondary metabolites to females during mating (e.g., Eisner & Meinwald, 1995), such as *Neopyrochroa flabellata*, the beetle that produces cantharidin (i.e., Spanish fly) (Eisner *et al.*, 1996).

Although not significant ($P = 0.073$), neonate larvae also appear to have some protection from predation. Bluegill consumed all 13 spotted salamander larvae but only 62% of the *Notophthalmus* neonates. This may be nothing more than a chemical "hangover" from any maternally-invested egg defenses, but as the neonate larvae of salamanders are—in general—extremely vulnerable to predation (Duellman & Trueb, 1994; Wells, 2007), any defensive capability is could be of adaptive advantage.

4.3 Eastern newts are chemically defended

Regardless of some disconnects between laboratory and field assays, eastern newts appear protected from many generalist consumers, and that protection is almost certainly chemical in nature. Most of the newts in the bass assay were spat out immediately, as were pieces of newt dorsum when fed to bluegill. Following rejection, both fishes repeatedly coughed and respired rapidly, likely in an attempt to flush their buccal cavity clean. Bluegill exhibited similar behaviors when crude chemical extracts of newts were imbedded in artificial foods, and newt eggs appear chemically protected as well (Figure 5), although we can not rule out mechanical defenses.

However, despite the production of TTX by eastern newts, we are aware of no attempt to trace the chemistry responsible through bioassay-guided fractionation and relevant consumers. Chemically defended organisms often use multiple toxic compounds in defense against consumers (Hay *et al.*, 1998). If we want to accurately predict how defensive chemistry affects the ecological and evolutionary trajectories of organisms, knowledge about all of the bioactive molecules present, and their interactions, is desirable. This is especially true when multiple defense compounds act synergistically. For example, the

seaweed *Halimeda goreaui* produces both an unusual secondary metabolite and is heavily calcified. Neither the metabolite nor $CaCO_3$ were individually effective at deterring urchin grazing; when combined, however, the synergy of chemical and structural traits were effective feeding deterrents (Hay *et al.*, 1994).

Although we were unsuccessful at isolating the chemical compounds responsible for predator deterrence in newts, our findings of activity in the polar fraction and a lack of activity in the lipophilic fraction (Figure 7) is congruent with the hypothesized role of TTX, a water-soluble molecule (Daly *et al.*, 1987; Zimmer & Ferrer, 2007). Our findings on the aversion response of naïve bluegill to relevant concentrations of TTX also lend support to the prevailing theory (Figures 8 and 9). Yet, despite the likelihood that TTX is the chemical responsible for most of the predator deterrence in eastern newts, other molecules could be involved. Most newt species produce tetrodotoxin derivatives, but we know much less about their efficacy and ecological impacts, or how the compounds interact with one another. Moreover, Yotsu-Yamashita & Mebs (2003) found that TTX concentrations in some *N. viridescens* efts were insufficient to explain the level of bioactivity seen in mice; the authors speculated on the existence of an additional deterrent compound but it was not identified.

4.4 Conclusions

There is little doubt that the tally of novel biochemical compounds isolated from amphibians will increase. Unfortunately, this information will be of limited value for ecologists until more efforts are made to connect the chemistry to the ecology and behaviors of the anurans, caudates, and caecilians that produce them. These connections are important to understand because amphibians are often key players of natural ecological systems, both in terms of biomass and impact. In many systems amphibians, especially salamander adults, are critically important apex predators, with some playing roles of 'keystone' consumers (Davic & Welsh, 2004; Wells, 2007). On a broader scale amphibians serve

as a critical link both among trophic levels and ecosystems. Some Appalachian salamander populations have an estimated caloric contribution of 1.165 kcal·m^2. Davic & Welsh (2004) put this energy impact in perspective by noting that the annual average harvest by the world's marine fishery is estimated at 0.3 kcal·m^2. As amphibian populations decline worldwide (Lannoo, 2005), research exploring the chemical ecology of consumptive interactions may help us find solutions to conservation issues such as the range expansion of cane toads (*Bufo marinus*) across Australasia, or help elucidate how to maximize amphibian biodiversity. For instance, although the eastern newt is not yet considered threatened, in fishless habitats *N. viridescens* serves as a critical mediator of the intense competition between several species of anuran and caudate tadpoles (see Wilbur, 1997, for review). Moreover, eastern newts are effective colonizers of a variety of manmade habitats like farm ponds, drainage ditches, and the 'remediated' wetlands adjacent to suburban strip malls—no doubt facilitated by their chemical defenses against predation (Petranka, 1998; Lannoo, 2005). Therefore, ensuring that newt populations remain healthy may help maximize the diversity of other amphibian species in the face of urban development. However, this requires a thorough understanding of the trophic relationships among organisms, chemically defended and not, if we are to do so.

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